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The *boule* gene is essential for spermatogenesis of haploid insect male

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ABSTRACT

boule (*bol*), a member of the *Deleted in Azoospermia* (*DAZ*) gene family plays an important role in meiosis (reductional maturation divisions) in a spermatogenesis-specific manner in animals by regulating translation of the downstream *cell division cycle 25* (*cdc25*) phosphatase mRNA. Orthologues of *bol* are conserved among animals and found in the genomes of hymenopteran insects, in which the general mode of reproduction is haplodiploidy: female is diploid and male is haploid. In this mode of reproduction, haploid males produce haploid sperm through non-reductional maturation divisions. The question thus arises of whether the *bol* gene actually functions during spermatogenesis in these haploid males. In this study, we identified two transcriptional isoforms of *bol* orthologue (*Ar bol* and *Ar bol*-2), and one *cdc25* orthologue (*Ar cdc25*) in the hymenopteran sawfly, *Athalia rosae*. *Ar bol* was expressed exclusively in the testis when maturation divisions occurred, while *Ar bol*-2 was expressed ubiquitously. Knockdown of all *bol* transcripts (both *Ar bol* and *Ar bol*-2) resulted in a lack of mature sperm, whereas males with sole knockdown of *Ar bol*-2 were able to produce a small number of mature sperm. The cell cycle was arrested before maturation divisions in the testis in which all *bol* transcripts were knocked down, as revealed by flow cytometry. Although no mature sperm was produced, sperm elongation was partially observed when *Ar cdc25* alone was knocked down. These results indicate that *Ar bol* is essential for the entry and progression of maturation divisions and sperm differentiation in haploid males.

Introduction

The *boule* (*bol*) gene is a member of the *Deleted in Azoospermia* (*DAZ*) gene family, which consists of *bol*, *DAZ-like* (*Dazl*), and *DAZ*. The most ancient member, *bol*, is conserved among nearly all metazoans. *Dazl* and *DAZ* arose by gene duplication and *Dazl* is conserved among vertebrates, while *DAZ* is found only in primates (Eberhart et al., 1996; Karashima et al., 2000; Shah et al., 2010; Xu et al., 2001). The *DAZ* family genes encode RNA-binding proteins bearing an RNP-type RNA-recognition motif (RRM) domain and a basic amino acid-rich *DAZ* repeat. They are considered to be involved in germ cell development in animals due to their conserved expression pattern specific to gametogenesis (Brook et al., 2009; VanGompel and Xu, 2011; Yen, 2004).

The *bol* gene was initially identified as the causative gene of meiotic entry defect mutation in males of *Drosophila melanogaster* (Castrillon et al., 1993). The *Drosophila* Bol protein is the translational regulator of *twine* (*twe*, a *cdc25* orthologue) mRNA (Maines and Wasserman, 1999), which promotes meiosis from G_2 to M phase in a testis-specific manner (Eberhart et al., 1996). Similarly, the *bol* orthologues of other animals so far examined are expressed in their testes and play crucial roles in spermatogenesis. An exception is the *bol* orthologue of *Caenorhabditis elegans*, which shows ovary-specific expression and is critical for oogenesis (Karashima et al., 2000; Shah et al., 2010).

The presence of multiple *bol* gene products derived from alternatively spliced isoforms and duplicated *bol* genes has been demonstrated in some animals. In the mouse, *Mus musculus*, one of the two alternatively spliced isoforms is expressed exclusively in spermatogenesis and the other in both spermatogenesis and oogenesis (Shah et al., 2010). The flatworm, *Macrostomum lignano*, has three *bol* paralogous genes. Two (*macbol*1 and *macbol*2) regulate spermatogenesis and the other (*macbol*3) regulates oogenesis (Kuales et al., 2011). In any case, *bol* is likely to be involved in the regulation of meiosis in animals (VanGompel and Xu, 2011). The functional exception of an alternatively spliced isoform is found in *D. melanogaster*, and this isoform contributes to development of the nervous system, but does not take part in germline development (Joiner and Wu, 2004).

Hymenoptera is a unique order of insects in which the general mode of reproduction is haplodiploidy (Crozier, 1977). Diploid females develop from fertilized eggs and haploid males develop from unfertilized eggs. Eggs are generated through meiosis (reductional maturation

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divisions) as in other animals. However, the maturation division during spermatogenesis is altered in haploid males. Ultrastructural and cytological observations of spermatogenesis in bees and wasps indicated that the reductional division in meiosis I is aborted, but the equational division (meiosis II) is maintained to produce haploid sperm (Sharma et al., 1961; Hoage and Kessel, 1968; Hogge and King, 1975; Cruz-Landim et al., 1980).

Recent progress in genome analysis has revealed the presence of meiosis-related genes in hymenopterans and other arthropods (Schurko et al., 2009; Schurko et al., 2010; The Honeybee Genome Sequencing Consortium, 2006; Werren et al., 2010). Schurko et al. (2010) found *bol* orthologues in the honeybee (*Apis mellifera*) and the jewel wasp (*Nasonia vitripennis*), in both of which canonical meiosis is absent in males. However, it is unknown whether this gene is actually expressed and functions in haploid males during spermatogenesis. To address these issues, we used the sawfly, *Athalia rosae*, which belongs to the most primitive members of Hymenoptera (Symphyta); the haploid males were shown to produce haploid sperm without reduction of the genome sets (Fujiwara et al., 2004; Naito and Suzuki, 1991). We identified and isolated the *bol* gene orthologue and examined its function in spermatogenesis by using gene knockdown methods established in this species (Sumitani et al., 2005; Yoshiyama et al., 2013). Our findings indicate that *bol* is required for the entry of non-reductional maturation divisions and sperm differentiation in haploid males. This is the first evidence demonstrating *bol* functions in haploid organisms.

Materials and methods

Sawfly

Laboratory stocks of the sawfly, *A. rosae*, were maintained at 25 °C under 16 h-light/8 h-dark conditions (Sawa et al., 1989). General biology, staging, and artificial induction of parthenogenetic development (egg activation) were described previously (Hatakeyama et al., 2000; Oishi et al., 1993; Sawa and Oishi, 1989).

Isolation of boule and cdc25 orthologues of A. rosae

The orthologues of *bol* and *cdc25* were obtained by PCR-based methods and high-throughput sequencing of mRNA. The cloning strategy and primers used are shown in Supplemental Table S1 and Fig. S1. Total RNA was extracted from the testes from the males of last-instar larval to late pupal stages and 48-h-old embryos using an RNeasy Mini kit (Qiagen). RACE-ready cDNA libraries were prepared using a SMART RACE cDNA library amplification kit (Clontech). An initial clone corresponding to an RNA recognition motif (RRM) was obtained by PCR using degenerate primers, and flanking 5′ and 3′ regions were obtained by RACE methods (Supplemental Fig. S1).

Libraries for high-throughput sequencing of mRNA were synthesized using a TruSeq RNA sample prep kit (Illumina). The libraries were sequenced by a single read method using a high-throughput DNA sequencer (HiSeq2000, Illumina). The fragments read (6.3×10^7 for the testicular library and 7.5×10^7 for the embryonic library) were assembled and finally gave rise to transcriptomes of 29,359 transcripts for the testis and 25,023 transcripts for the embryo. The orthologous sequences of *bol*/*Dazl*/*Daz* and *cdc25* were searched using a BLAST program (Altschul et al., 1997) supplied with the GENETYX bioinformatics software (GENETYX Corporation).

Genomic DNA was extracted from 1-day-old adult males using a DNeasy blood & tissue kit (Qiagen). Genomic regions corresponding to the *Ar bol* gene and the *Ar cdc25* gene were amplified by PCR using gene-specific primer sets. Each PCR product was examined by agarose gel electrophoresis, purified, and cloned into the pCR 2.1 vector using a TOPO TA cloning kit (Invitrogen). Cloned cDNA fragments were sequenced and analyzed with a DNA sequencer (Applied Biosystems, ABI 3130xl).

Molecular phylogenetic analyses

Homology search and multiple alignments of the sequences were performed using BLAST and Clustal W (Thompson et al., 1994) supplied in MEGA5 software (Tamura et al., 2011). The sequence data used for analyses are listed in Supplemental Table S2. Phylogenetic analyses based on the deduced amino acid sequences were performed by Bayesian inference (BI) methods using MrBayes v3.1.2 software (Huelsenbeck and Ronquist, 2001). The optimal models of amino acid substitution were selected using the corrected AIC in Aminosan (Tanabe, 2011) as LG Gamma and VT+F Gamma models for *bol* and *cdc25*, respectively. The BI analyses were performed by setting the number of Markov chain Monte Carlo (MCMC) generations at three million, the sampling frequency at 1000, and calculating a consensus topology from a pool of 10,001 trees after discarding the first 10,000. Statistical support for the resultant BI trees was determined with Bayesian posterior probabilities (BPP).

Reverse transcription PCR (RT-PCR)

Total RNAs used as the template were extracted from embryos at 0 h, 2 days, 3 days, and 5 days after egg activation, larvae, male and female adults devoid of gonads, ovaries, and testes using an RNeasy Mini kit (Qiagen). RT-PCR to amplify a part of the transcripts of *Ar bol*, *Ar bol*-2, *Ar cdc25*, and *A. rosae elongation factor-1α* (*Ar ef-1α*: GenBank accession no. AB253792) was performed using a One-step RT-PCR kit (Qiagen) according to the supplier's protocol. The primer set of Arbol-F3 and Arbol-R2 was designed to amplify fragments distinguishable for the *Ar bol* transcript (521 bp) and *Ar bol*-2 transcript (1439 bp). The primer set of Arbol-F3 and Arbol-R3 was for a fragment specific to the *Ar bol*-2 transcript (599 bp). The primer sets of Arcdc25 F3 and Arcdc25 R3, and ArEF1-LP and ArEF1-RP amplify a part of the *Arcdc25* (550 bp) and *Ar ef-1α* (313 bp) transcripts, respectively (Supplemental Table S1 and Fig. S1). The number of PCR cycles was 25 for all amplifications.

Quantitative RT-PCR (qRT-PCR) was performed by two-step methods. Total RNA extracted from testes of early pupae was treated with DNase and reverse-transcribed to cDNA using a PrimeScript RT reagent kit (Takara). The cDNAs were subjected to PCR using SYBR Premix Ex Taq (Takara) and the primer sets described above. Serial dilutions of a plasmid containing a fragment of each transcript were used as a standard. Reactions were analyzed with a real-time PCR system (LightCycler 480, Roche) under the conditions of 95 °C for 5 min, followed by 45 cycles of 95 °C for 5 s and 62 °C for 30 s. The results were normalized against *Ar ef-1α* transcript.

Gene knockdown by RNA interference (RNAi)

Short cDNA fragments corresponding to *Ar bol* and *Ar cdc25* were amplified using PrimeSTAR HS DNA Polymerase (Takara) and primer sets of dsRNA Arbol F1 and dsRNA Arbol R1 (common to *Ar bol* and *Ar bol*-2, 250 bp), dsRNA Arbol F2 and dsRNA Arbol R2 (specific to *Ar bol*-2, 280 bp), and dsRNA Arcdc25 F and dsRNA Arcdc25 R (320 bp) (Supplemental Table S1 and Fig. S1). The PCR product was flanked by the T7 polymerase promoter sequence that is incorporated at the 5′ end of each primer. The double-stranded RNAs (dsRNAs) were synthesized using an Ambion MEGAscript T7 kit (Ambion) and the cDNA fragments as templates. The locations of each dsRNA are indicated in Fig. 1A and C. It is not possible to synthesize a dsRNA specific to the *Ar bol* isoform due to the structure of the transcripts. Synthesized dsRNA was adjusted to a concentration of 1 μg/μl, and injected at 3 μg per individual to the

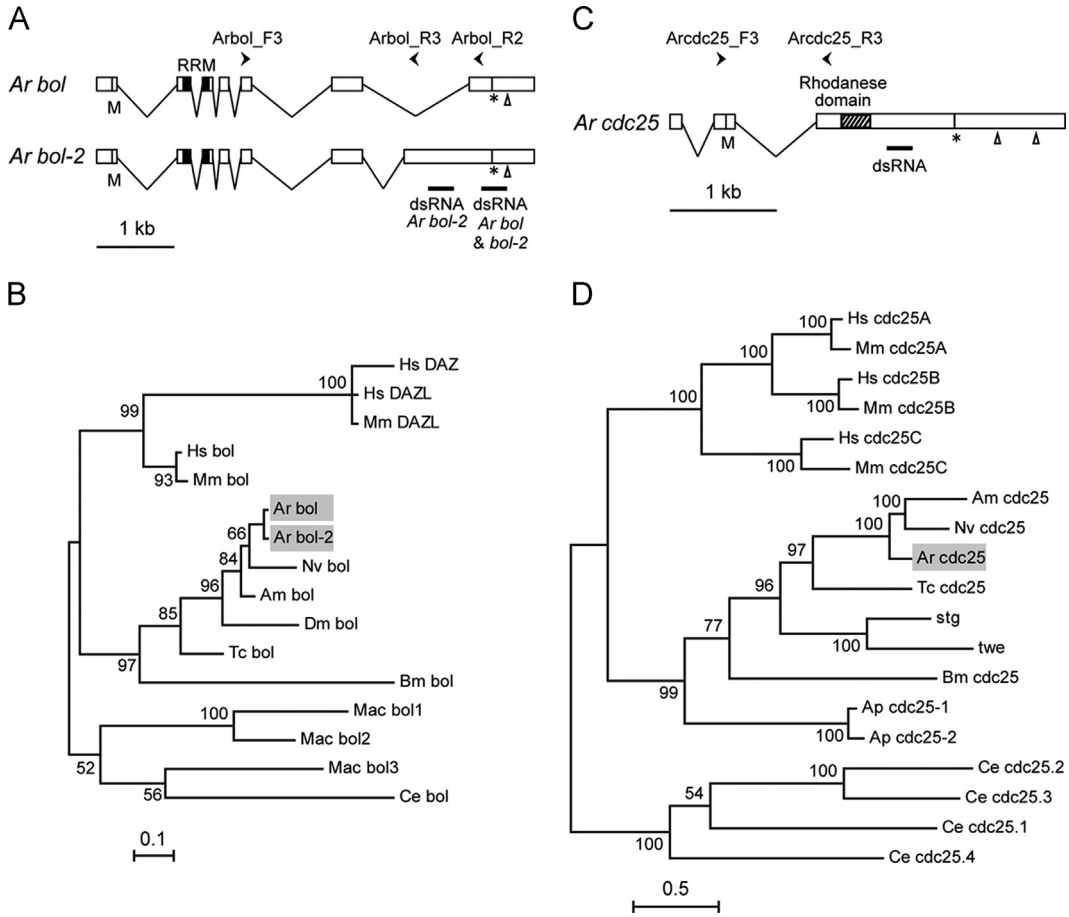

Fig. 1. Characterization of the transcripts of *A. rosae bol* (*Ar bol*) and *cdc25* (*Ar cdc25*). Structures of the transcriptional variants of *Ar bol* and *Ar bol*-2 (A), and the *Ar cdc25* transcript (C). Open boxes, solid boxes, and hatched boxes indicate exons, RNA recognition motif (RRM), and the Rhodanese domain, respectively. The initial methionine and the stop codon are marked by M and an asterisk, respectively. Solid lines on the bottom indicate the regions corresponding to where double-stranded RNA (dsRNA) is synthesized. Triangles indicate the position of a presumed polyadenylation signal (AATAAA). Primers used for RT-PCR to amplify fragments of the *Ar bol* and the *Ar bol*-2 transcripts (Arbol F3 and Arbol R2), and a fragment specific to the *Ar bol-2* transcript (Arbol F3 and Arbol R3) are indicated by arrowheads. It is not possible to synthesize a dsRNA specific to the *Ar bol* transcript owing to its structure. Molecular phylogenetic tree constructed by Bayesian methods using the amino acid sequences corresponding to the RRM domains of *DAZ* family genes (B) and the entire amino acid sequences of *cdc25* gene orthologues (D). Bootstrap values are indicated at branching points. The scale indicates the number of amino acid substitutions per site. The sequence data used for analyses are listed in Supplemental Table S2. Am: *Apis mellifera*, Ap: *Acyrthosiphon pisum*, Bm: *Bombyx mori*, Ce: *Caenorhabditis elegans*, Dm: *Drosophila melanogaster*, Hs: *Homo sapiens*, Mac: *Macrostomum lignano*, Mm: *Mus musculus*, Nv: *Nasonia vitripennis*, Tc: *Tribolium castaneum*, stg: *string*, and twe: *twine*.

last-instar larvae (larval RNAi) or newly emerged adults (parental RNAi) anesthetized by chilling on ice for 0.5–1 h, as described by Yoshiyama et al. (2013).

Microscopy

Gonads and internal reproductive organs dissected from larvae, pupae and adults, and embryos were observed and photographed under a stereomicroscope (Leica, MZ16F).

Larval, prepupal, pupal, and adult testes were fixed with a few drops of 45% acetic acid and stained with lactic–acetic–orcein solution (lactic acid:acetic-orcein [3% orcein+60% acetic acid]= 1:1) on microscope slides. The specimens were covered immediately with coverslips, examined, and photographed under a microscope (Axio Imager A1, Carl Zeiss) equipped with a CCD camera (AxioCam MRc5, Carl Zeiss).

Flow cytometry

Fluorescent-labeled cell samples were prepared based on the methods described by Pozarowski and Darzynkiewicz (2004) with modifications. Testes of 3–5 individuals placed in a 1.5 ml micro-centrifuge tube with 100 μl of PBS (phosphate-buffered saline, pH 7.4) solution were homogenized using a plastic pestle and treated with trypsin at a final concentration of 0.1–0.2% in PBS for 30 min to disaggregate the cells. After brief centrifugation (380*g*) to remove debris, the solution was replaced with HBSS (Hanks' balanced salt solution, Gibco) and then stained with Hoechst 33342 (Sigma) at a final concentration of 5 μg/ml for 1 h. The cell-containing solution was replaced with fresh HBSS and filtered using a cell strainer with a 35 μm mesh (BD Falcon). The cell samples were analyzed with a flow cytometer (MoFlo Astrios, Beckman-Coulter).

Results

Identification and characterization of the sawfly bol and cdc25 gene orthologues

We obtained two isoforms of *A. rosae boule* transcripts: *Ar bol* (GenBank accession no. AB719980) from the testicular cDNA library and *Ar bol*-2 (GenBank accession no. AB719981) from the embryonic cDNA library (Fig. 1A). The full-length cDNAs of *Ar bol* and *Ar bol*-2 were 2651 bp and 3569 bp, encoding 487 and 793 amino acids, respectively. Both *Ar bol* and *Ar bol*-2 isoforms consisted of seven exons, differing in the last exon due to an alternative

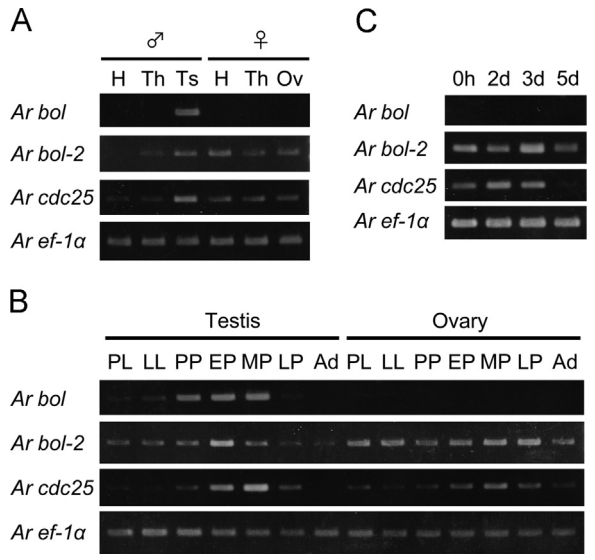

Fig. 2. Expression of *Ar bol*, *Ar bol*-2, and *Ar cdc25* analyzed by RT-PCR. (A) Somatic tissues and gonads of male and female in the early pupal stage. *Ar bol* is expressed exclusively in testes. *Ar bol*-2 and *Ar cdc25* are expressed in somatic tissues and gonads. H: heads, Th: thoraxes, Ts: testes, Ov: ovaries. *Ar ef-1α* is the positive control. (B) Temporal expression patterns of each gene in testes and ovaries. *Ar bol* is expressed in testes from the last-instar larval to mid-pupal stages. *Ar bol*-2 is expressed in both testes and ovaries. *Ar cdc25* is expressed in testes from prepupal to late pupal stages and ovaries in all stages. PL: penultimate-instar larvae, LL: last-instar larvae, PP: prepupae, EP: early pupae (immediately after pupation), MP: mid-pupae (2 days after pupation), LP: late pupae (4 days after pupation), Ad: adult (immediately after eclosion). (C) Expression of each gene during embryonic development. No expression of *Ar bol* is detected during embryogenesis, while *Ar bol*-2 is expressed at all stages. *Ar cdc25* is expressed at all stages except for the last day of embryogenesis. 0 h, 2 d, 3 d, and 5 d indicate hours and days after parthenogenetic development begins.

3′ splicing site in intron 6 (Fig. 1A). All intronic regions were spliced according to the GU–AG rule. The deduced amino acid sequence of each isoform had the characteristic domain representing *DAZ* family members, an RNA recognition motif (RRM) near the N-terminal, which is very similar to those of Bol, Dazl, and DAZ of other animals so far identified (Supplemental Fig. S2A). Both *Ar* Bol and *Ar* Bol-2 lacked the DAZ repeat. The DAZ repeat is absent in the Bol proteins of other insects, while the motif is present in Bol of *D. melanogaster*. Molecular phylogeny of the *DAZ* family based on the RRMs revealed that *Ar* Bol and *Ar* Bol-2 were placed in the strongly supported monophyletic clade of hymenopteran Bol molecular species (Fig. 1B). The genomic region of the *Ar bol* gene (GenBank accession no. AB719982) was obtained by PCR-based methods. *Ar bol* was shown to be a single-copy gene, which was confirmed by examining the *A. rosae* genome sequence (GenBank accession nos. KB467405–KB467808) and by Southern blot analysis (Supplemental Fig. S2B).

The *A. rosae cdc25* (*Ar cdc25*) orthologue (GenBank accession no. AB910530) obtained from the testicular cDNA library was 2747 bp in length, encoding 494 amino acids (Fig. 1C). Comparison of *Ar cdc25* cDNA and the genomic region revealed that the 5′ untranslated region (UTR) and the ORF, respectively, were interrupted by an intron according to the GU–AG rule (Fig. 1C). The Rhodanese domain containing the HC motif (IVXHCXXXXXR), a characteristic sequence in all phosphotyrosyl phosphatases (Millar and Russell, 1992), was present. There were two GUUC sequences in the 3′ UTR, the putative Bol binding sites (Maegawa et al., 2002). The phylogeny based on the entire *cdc25* amino acid sequences showed that *Ar* Cdc25 was placed in the strongly supported monophyletic clade of hymenopteran Cdc25, related to the Cdc25 orthologues (String and Twine) of *D. melanogaster* (Fig. 1D). It was confirmed by examining the *A. rosae* genome and by Southern blot analysis (Supplemental Fig. S2C) that *Ar cdc25* (GenBank accession no. AB910531) is a single-copy gene.

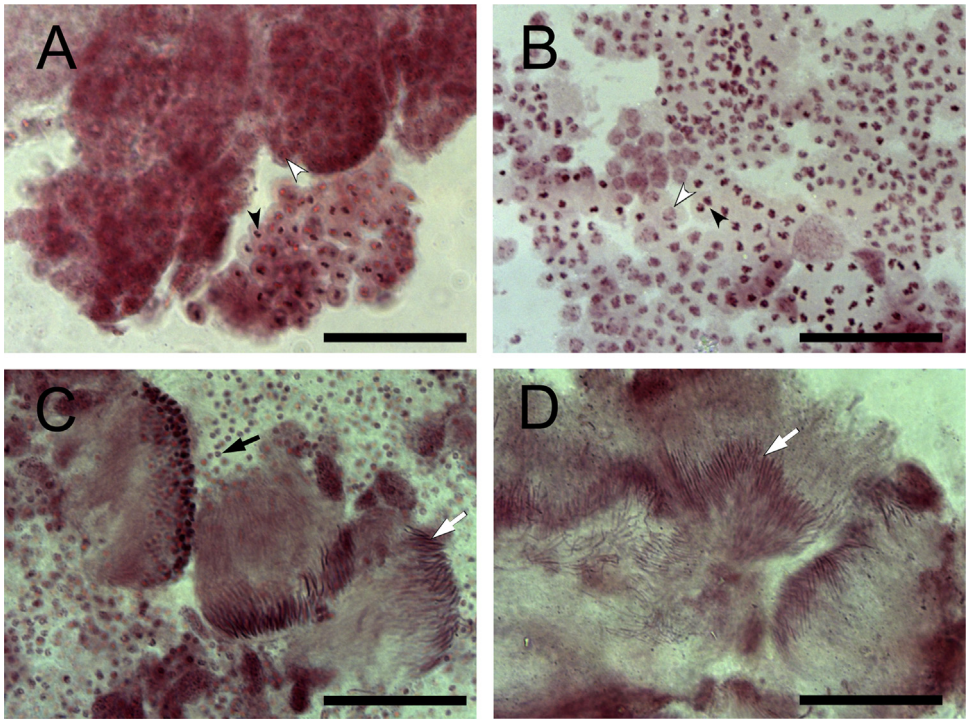

Fig. 3. Lactic–acetic–orcein-stained testicular preparations of normal wild-type males. (A) Prepupal testes (one day before pupation). Some condensed chromosomes are observed in spermatocytes showing the initiation of maturation divisions. (B) Early pupal testes (immediately after pupation). Maturation divisions progress in many spermatocytes. (C) Pupal testes (one day after pupation). Round spermatids are found and some of them enter spermiogenesis, as seen by the cells beginning to elongate. (D) Mid-pupal testes (three days after pupation). Many elongating and elongated spermatids are found and sperm bundle-like shapes are seen in some cysts. Spermatocytes before the maturation division, dividing spermatocytes, round spermatids, and elongating spermatids are indicated by open arrowheads, solid arrowheads, solid arrows, and open arrows, respectively. Bars indicate 50 μm.

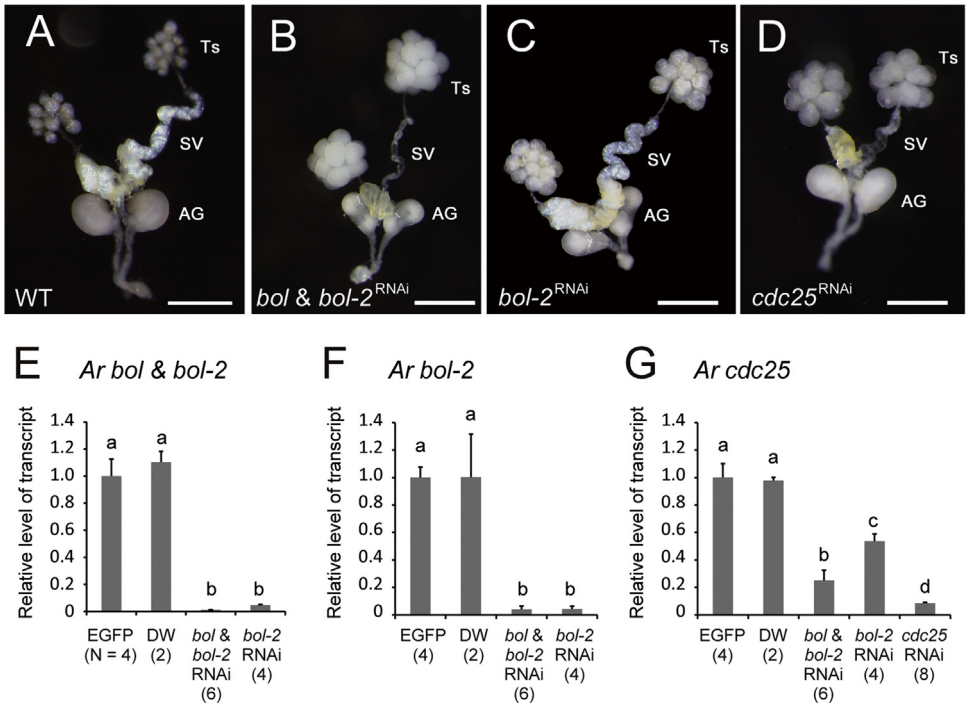

Fig. 4. Morphology of testes and internal reproductive organs taken from wild-type and gene knockdown male adults and expression of targeted genes. (A) Testes shrink considerably and mature sperm are transferred to and stored in the seminal vesicles in 3-day-old wild-type male. (B) Testes do not shrink and no mature sperm are detected in seminal vesicles when both *Ar bol* and *Ar bol*-2 are knocked down (*bol* & *bol*-2^{RNAi}). (C) Testes shrink to some degree and mature sperm are observed in the seminal vesicle when *Ar bol*-2 alone is knocked down (*bol*-2^{RNAi}). (D) A similar phenotype compared to that of *bol* & *bol*-2^{RNAi} is observed when *Ar cdc25* is knocked down (*cdc25*^{RNAi}). Ts: testes, SV: seminal vesicle, AG: accessory gland. One of the seminal vesicles is stretched to show accumulation of mature sperm. Bars indicate 500 μm. Histograms show relative levels of transcripts of total *Ar bol* (*Ar bol* & *bol*-2) (E), *Ar bol*-2 (F), and *Ar cdc25* (G) in each testis determined by qRT-PCR. The level of transcript in the control testes with which dsRNA corresponding to a short fragment of the *enhanced green fluorescent protein* (*EGFP*) gene (Yoshiyama et al., 2013) is injected is set as 1. Each value and error bar indicates the group mean and standard deviation, respectively. Different letters above bars represent significant differences at $p < 0.001$ (Student's *t* test with Bonferroni correction). DW: distilled water injection.

Table 1
Knockdown effects of *Ar bol*, *Ar bol*-2, and *Ar cdc25* on spermatogenesis.

Target transcripts	Dose (μg/larva)	No. of larvae injected	No. of adult males survived/examined	Sperm in seminal vesicles	
				Absent completely	Present
bol & *bol*-2	3 μg	38	13	13	0
bol-2 alone	3 μg	37	12	2	10
cdc25	3 μg	14	3	3	0
bol & *bol*-2, *cdc25*	3 μg each	7	3	3	0
RNase-free water	–	30	6	0	6

Expression patterns of Ar bol, Ar bol-2, and Ar cdc25

Ar bol was expressed exclusively in testis (Fig. 2A) and the expression began at the last-instar larval stage and lasted until the mid-pupal stage (Fig. 2B). Cytological observation revealed that spermatocytes were predominant in the prepupal testes and a fraction of them began dividing (Fig. 3A). Immediately after pupation, the majority of the cells in testes was round in shape (round spermatid) (Fig. 3B). Round spermatids then started to elongate (Fig. 3C) and almost all spermatids had fully elongated to form sperm bundles in the mid-pupal testes (Fig. 3D). These observations are in line with a previous report (Hatakeyama et al., 2000). The expression of *Ar bol*, therefore, coincided with the progression of maturation divisions and the sperm differentiation. In contrast, *Ar bol*-2 was expressed not only in testes, but also in ovaries and somatic tissues, such as head and thorax, in the pupal stage (Fig. 2A). *Ar bol*-2 expression was seen in the testes from the penultimate-instar larval to adult stages, although the level of expression was low, except for in the early pupal stage (Fig. 2B). *Ar bol*-2 expression was also detected in the ovaries in all stages examined and during embryogenesis (Fig. 2C).

Ar cdc25 expression was detected in testes and ovaries as well as somatic tissues in the early pupal stage (Fig. 2A). The expression in the testes appeared from the prepupal to late pupal stages, with its peak expression in the mid-pupal stage (Fig. 2B). A similar expression pattern was seen in the ovaries. It was expressed throughout embryogenesis, except for the stage immediately before larval hatching (Fig. 2C).

Functional analysis of Ar bol, Ar bol-2, and Ar cdc25 by RNAi

Ar bol, *Ar bol*-2, and *Ar cdc25* transcripts were silenced by injection with dsRNAs into the last-instar larvae, after which the phenotypes were examined. In the normal 3-day-old adult males, large numbers of mature sperm in the form of sperm bundles were transferred and stored in the seminal vesicles, and the testes shrank (Fig. 4A). When both *Ar bol* and *Ar bol*-2 were knocked

down, the level of these transcripts markedly decreased in the pupal stage (Fig. 4E and F). It should be noted that the level of *Ar cdc25* transcript was affected by knockdown of both *Ar bol* transcripts (Fig. 4G). No sperm bundle was observed in the seminal vesicles and the testes looked intact in these knockdown male adults (Table 1, Supplemental Table S3, and Fig. 4B). In the testes of these knockdown male adults, spermatocytes and round spermatids predominated (Fig. 5C), as in the early pupal testes of wild-type males (Fig. 3B). The knockdown males copulated normally with females, while no fertilized diploid individuals (females) were obtained (Table 2). Injection of dsRNA specific to *Ar bol*-2 resulted in a marked decrease of the level of *Ar bol*-2 transcript (Fig. 4E and F). The phenotypes of the *Ar bol*-2-knockdown adult males varied: mature sperms forming bundles were present in the seminal vesicles of many knockdown males (Table 1, Supplemental Table S3, and Fig. 4C). Despite the presence of the sperm bundles in the seminal vesicles, the testes shrank only slightly (Fig. 4C) and had round or elongating spermatids (Fig. 5D), as seen in the pupal testes (Fig. 3C). These *Ar bol*-2-knockdown males were fertile since they contributed to fertilize eggs as diploid individuals (females) were obtained (Table 2). These results indicate that both *Ar bol* and *Ar bol*-2 are required for the completion of spermatogenesis, but *Ar bol*-2 seems to be less important than *Ar bol* for this.

Knockdown of *Ar cdc25* resulted in a phenotype with slightly shrunken testes and the absence of mature sperm bundles in the seminal vesicles (Table 1, Supplemental Table S3, and Fig. 4D),

Table 2
Knockdown effects of *Ar bol*, *Ar bol*-2, and *Ar cdc25* on male fertility.

Transcripts knocked down	No. of offspring examined	Progeny	
		Females (fertilized)	Males (parthenogenesis)
bol & *bol*-2 ($n=3$)	57	0	57
bol-2 alone ($n=4$)	150	53	97
cdc25 ($n=3$)	38	0	38
None ($n=3$)	94	53	41

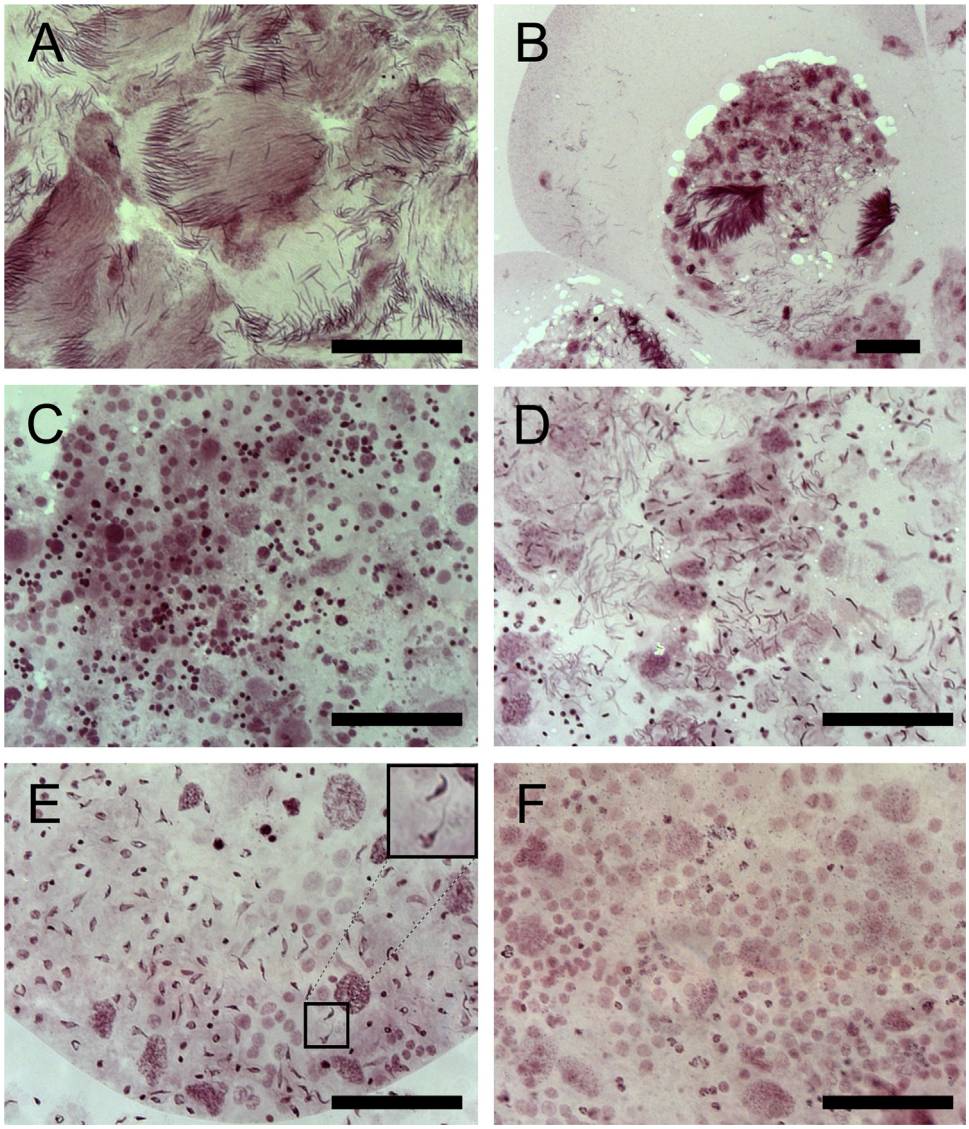

Fig. 5. Phenotypes of testicular cells in gene knockdown male adults. (A) Most of the cells complete spermatogenesis and mature sperm in bundles are formed in the testes of wild-type males immediately after eclosion. (B) Testicular cysts are almost empty due to the transfer of mature sperm into seminal vesicles in the 2-day-old adults. (C) Both *Ar bol* and *Ar bol*-2 knockdown males after three days of eclosion. Round cells predominate in the testes and no elongating spermatids are found. (D) Testes of *Ar bol*-2 solely knocked down males after four days of eclosion. Round cells, elongated spermatids, and mature sperm are observed. (E) *Ar cdc25*-knockdown male testes after four days of eclosion. Round cells and elongating cells are observed. Inset shows a magnified image. (F) All cells are round in shape in the testes after two days of eclosion when *Ar bol*, *Ar bol*-2, and *Ar cdc25* are all knocked down. Bars indicate 50 μm.

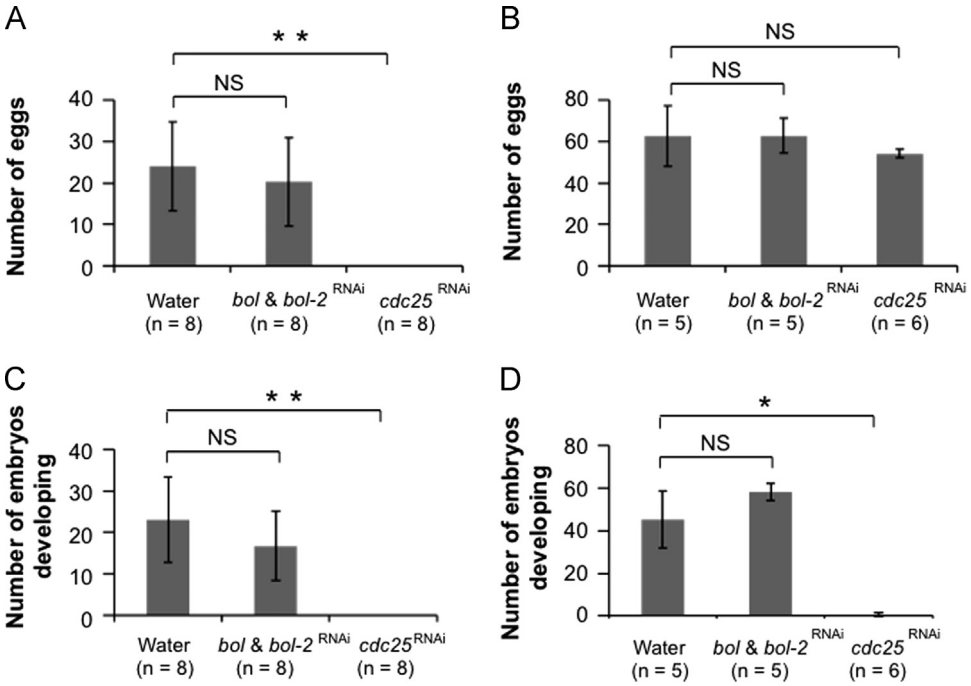

Fig. 6. Effects of knockdown of *Ar bol*, *Ar bol*-2, and *Ar cdc25* in females. Effects of larval injection (A) and parental injection (B) with the dsRNAs corresponding to both *Ar bol* and *Ar bol*-2, and *Ar cdc25* on female fecundity. Larval injection of *Ar cdc25* dsRNA significantly reduces the number of eggs that mature in the ovaries (**: Mann–Whitney *U* test with Bonferroni correction $p < 0.001$). Numbers of embryos normally developing from the eggs taken from females that received larval injection (C) and parental injection (D) of the dsRNAs targeting both *Ar bol* and *Ar bol*-2 or *Ar cdc25* (*: Mann–Whitney *U* test with Bonferroni correction $p < 0.05$).

similar to those observed in all *Ar bol*-knockdown phenotypes. Nevertheless, elongating cells were observed in the testes (Fig. 5E).

Females that underwent larval injection with the dsRNA targeting both *Ar bol* and *Ar bol*-2 transcripts showed a marked decrease of ubiquitously expressed *Ar bol*-2 transcript in the ovaries (Supplemental Fig. S3), while having normally developed ovaries with an ordinary number of mature-looking eggs (Fig. 6A). Similarly, parental injection of *Ar bol* dsRNA showed no effects on the reproductive abilities of females (Fig. 6B). Eggs produced by these knockdown females developed to haploid males upon artificial activation (Fig. 6C and D). In addition, these male progeny were fertile, having normal testes and mature sperm in the seminal vesicles (data not shown).

On the other hand, knockdown of *Ar cdc25* was detrimental to females. No eggs matured in adults when *Ar cdc25* dsRNA was injected into the last-instar larvae (Fig. 6A). Although mature-looking eggs were produced in the case of parental injection (Fig. 6B), nearly all of them failed to develop (Fig. 6D).

Effects of knockdown of Ar bol, Ar bol-2, and Ar cdc25 on maturation divisions

The progression of the cell cycle during spermatogenesis was evaluated by measuring the amount of DNA in a cell by flow cytometry analysis (Fig. 7). In normal spermatogenesis, a single peak of cellular DNA content was detected in the last-instar larval and prepupal testicular cells, and the proportion of cells containing half the amount of DNA compared with these testicular cells increased in early pupae (Fig. 7A). In the mid-pupal stage, the cells with half the amount of DNA dominated in the testicular cells. It could be interpreted that diploid (2N) cells (G_2 or before the M phase) were dominant in the last-instar larval and prepupal testes, haploid (N) cells (after the M phase) appeared and increased in early pupal testes, and finally haploid (N) cells dominated in the testes in the mid-pupal stage. These results agree well with the cytological observation (Fig. 3) and a previous description (Hatakeyama et al., 2000): round spermatids that had undergone maturation divisions were predominant in the testes of just-pupated males and most of the cells completed maturation divisions to reach a haploid state and proceeded to spermiogenesis in the mid-pupal stages.

In the testes of males in which both *Ar bol* and *Ar bol*-2 were knocked down, predominant diploid cells in prepupal stages maintained their diploid state thereafter (Fig. 7B), indicating that cell cycles were arrested at G_2 or before the M phase of maturation divisions. When *Ar bol*-2 alone was knocked down, a fraction of haploid cells appeared in the early pupal testes, although the proportion was not increased and diploid cells remained in the mid-pupal testes (Fig. 7C). In the testes of *Ar cdc25*-knockdown males, diploid cells were maintained from the prepupal to mid-pupal stages, similar to the results observed when both *Ar bol* isoforms were knocked down (Fig. 7D). Taking these results together, *Ar bol* and *Ar cdc25* are critical for the entry and progression of maturation divisions, *Ar bol*-2 is less important for the completion of maturation divisions, and both *Ar bol* isoforms are required for sperm differentiation.

Discussion

Several reports indicate that the *DAZ* family genes are required for the progression of meiosis and the differentiation of sperm in animals (reviews: White-Cooper, 2010; Shah et al., 2010; VanGompel and Xu, 2011). An issue that arises in this context is the function of an ancestral member of the gene family, *boule* (*bol*), found in the genomes of hymenopteran insects (Schurko et al., 2010), because the general mode of reproduction is haplodiploidy in Hymenoptera: males are haploid, so sperm is produced by non-reductional maturation divisions (Crozier, 1977). In this study, we addressed the issue of whether *bol* functions in haploid males of hymenopteran insects using the sawfly, *Athalia rosae*. After identification of the *bol* orthologue, expression analysis, knockdown experiments, and flow cytometry of testicular cells, it was revealed that the *bol* gene products are crucial for the completion of spermatogenesis

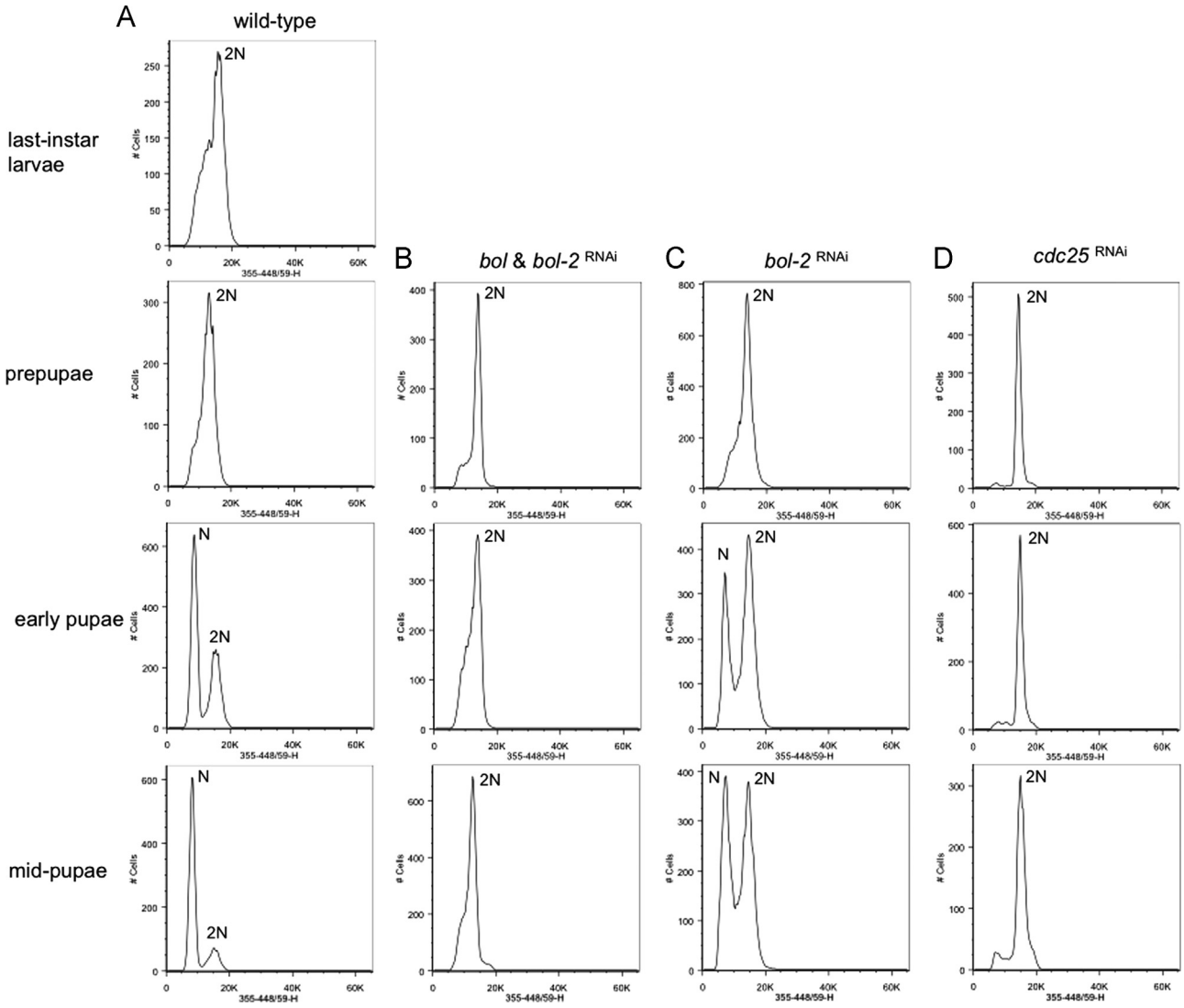

Fig. 7. Flow cytometry analysis of testicular cells. Histogram of DNA contents of the testicular cells prepared from the last-instar larvae, prepupae, early pupae, and mid-pupae for the (A) wild type, (B) both *Ar bol* and *Ar bol*-2 knockdown (*bol* & *bol*-2^{RNAi}), (C) *Ar bol*-2 knockdown alone (*bol*-2^{RNAi}), and (D) *Ar cdc25* knockdown ($cdc25^{RNAi}$).

in haploid males, but affect neither oogenesis in diploid females nor development in both sexes.

Two alternatively spliced transcripts (*Ar bol* and *Ar bol*-2) derived from a single *bol* gene were identified in *A. rosae*. Knockdown of both *Ar bol* transcripts resulted in global arrest of spermatogenesis and a lack of mature sperm. The phenotype resembled the loss-of-function of *bol* in *Drosophila* (Eberhart et al., 1996) and mouse (Shah et al., 2010). The present results indicate that *A. rosae bol* gene products are required for both the progression of maturation divisions, namely, the G_2/M transition, and sperm differentiation. It is assumed in *Drosophila* that the *bol* gene products have dual functions in G_2/M transition and sperm differentiation. The defect of spermatogenesis in the *bol* loss-of-function individuals is primarily caused by failure of the G_2/M transition, leading to the absence of sperm and male sterility (Eberhart et al., 1996). On the other hand, sperm differentiation occurs in the *twine* (*twe*, a *cdc25* orthologue) mutant testes, in which spermatocytes do not complete meiosis and remain in their 4N state (Alphey et al., 1992; Courtot et al., 1992; Lin et al., 1996; White-Cooper et al., 1993). The *cdc25*-knockdown phenotype of *A. rosae* showed nuclear elongation of testicular cells, as observed in the *twe* mutants in *Drosophila*. Sperm differentiation is, therefore, independent of the completion of maturation (meiotic) divisions in these two insects. The *bol* gene products apparently contribute to regulate these two consecutively occurring events during spermatogenesis. It is presumed that Bol regulates the translation of *cdc25* mRNA in the pathway of G_2/M transition in the sawfly, considering the conserved function of Bol and the shared structure of Bol-binding sequences in *Ar cdc25* mRNA. However, the involvement of Bol in the expression of *Ar cdc25* or stabilization of its transcript could not be ruled out since the level of *Ar cdc25* transcript was affected by *Ar bol* knockdown.

Crosstalk between the pathway of G_2/M transition and sperm differentiation is suggested after extensive investigations of meiotic-arrest genes and spermatid-differentiation genes in *Drosophila* (Bergner et al., 2010; Mikhaylova et al., 2006; White-Cooper, 2010). Translation of *modulo* (*mod*), a transcriptional activator of sperm-differentiation genes in testis, is controlled by Bol in *Drosophila* (Mikhaylova et al., 2006). The *mod* gene is conserved among drosophilids, while no homologs have been found in other animals including insects and the sawfly, *A. rosae* (data not shown). It remains to be seen what factor(s) is under the control of Bol in the pathway of sperm differentiation other than in *Drosophila*.

In the sawfly, one of the isoforms (*Ar bol*) plays a crucial role in spermatogenesis, while the other (*Ar bol*-2) might have subsidiary effects. When *Ar bol*-2 was solely knocked down, spermatogenesis

was severely interfered with, although a markedly reduced number of sperm was produced and these males were fertile. Considering the expression patterns of the sawfly *bol* isoforms: exclusive expression of *Ar bol* in testis when maturation divisions take place versus ubiquitous expression of *Ar bol*-2, it is assumed that there are functional differences between these isoforms. Apparent functional divergence among the *bol* splicing isoforms is reported in *Drosophila*. One isoform expressed exclusively in testis plays key roles in the correct progression of meiosis and spermiogenesis (Cheng et al., 1998; Maines and Wasserman, 1999; Mikhaylova et al., 2006), while an isoform that shows non-testis-specific expression patterns is required for development of the nervous systems (Joiner and Wu, 2004). In contrast, the contribution of both splicing isoforms of the sawfly is absolutely specific to spermatogenesis. Females were not affected at all by the knockdown of both *Ar bol* isoforms. Embryogenesis was completed and normal germ cells were produced devoid of *Ar bol* transcripts. It is thus unlikely that the sawfly *bol* isoforms function outside of spermatogenesis. Although we have not yet discriminated each of their functions, it is possible that *Ar bol* and *Ar bol*-2 interact with each other or with other proteins, since a characteristic of *DAZ* family gene products is binding to a variety of protein partners to mediate specific functions in vertebrates (review: Brook et al., 2009; VanGompel and Xu, 2011). Further studies will be required to identify binding partners and downstream factors in the sawfly.

In contrast to the dual functions of sawfly *bol* products, spermatogenic arrest occurred at postmeiotic, round spermatid stages in the *bol*-knockout mice, and mouse *bol* did not seem to be involved in the translation of *cdc25* mRNA (VanGompel and Xu, 2010). The authors consider that the role of mouse *bol* is specialized in sperm differentiation. It is proposed that *bol* and the duplicated gene, *Dazl*, redundantly regulate spermatogenesis and a possible role of *Dazl* is to compensate for the loss of *bol* in mice (Shah et al., 2010; VanGompel and Xu, 2011). The *Dazl* genes of several animal species so far examined show broad expression patterns, suggesting their multiple roles (reviews: Eirin-Lopez and Ausio, 2011; VanGompel and Xu, 2011). It has been demonstrated that the *Dazl* gene products are required for the development of primordial germ cells (PGCs) in mouse, frog (*Xenopus laevis*), and zebrafish (*Danio rerio*) (Houston and King, 2000; Lin and Page, 2005; Maegawa et al., 1999). Intriguingly, in the flatworm (*Macrostomum lignano*), one of the three *bol* paralogues termed *macbol3* is expressed in an ovary-specific manner and is essential for oogenesis, while neither spermatogenesis nor PGC development is affected by it (Kuales et al., 2011). On the other hand, the dual functions of *bol* are presumably maintained and conserved in the organisms that have only the ancestral *bol* gene, since the coordination of onset of maturation divisions with sperm differentiation is indeed the primary function of the *bol* gene.

Interestingly, the hymenopteran insects examined to date have only one *cdc25* orthologue (Schurko et al., 2010), in contrast to *D. melanogaster*, which has two: *twine* for entry into meiosis and *string* for mitosis (Alphey et al., 1992; Courtot et al., 1992). The pea aphid, *Acyrthosiphon pisum*, has at least two *cdc25* orthologues caused by aphid-specific gene duplication, which occurred recently (Srinivasan et al., 2010). Three *cdc25* genes are found in mammals (Karlsson-Rosenthal and Millar, 2006). Although *cdc25* is a functionally conserved gene, it has been frequently and independently duplicated in various animals. Hymenopteran *cdc25* might retain its primitive functions, although this insect group has a peculiar reproduction system, haplodiploidy.

Haplodiploidy represented by the hymenopteran insects has independently originated numerous times in animals and this reproduction system has been repeatedly acquired in insect evolution (Mable and Otto, 1998). Nevertheless, functions of the ancestral *bol* gene were conserved in the haploid sawfly males. As reviewed by Eirin-Lopez and Ausio (2011), functional conservation of the *DAZ* family genes across animal lineages suggests that spermatogenesis shares a common evolutionary origin derived from an ancestral prototype. A similar assumption can be made about oogenesis. We have demonstrated the function of the *c-mos* gene product, Mos, as a cytostatic factor to achieve meiotic arrest crucial to oocyte maturation in the sawfly (Yamamoto et al., 2008). Shared structures and functions of Mos in animals so far examined (Nishiyama et al., 2010) imply that oogenesis in terms of oocyte maturation seems to have evolved from a common origin. Further investigations of these factors that are tightly coupled to and controlled by sex will help us to understand how sexual reproduction has evolved.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.12.027.

References

Alphey, L., Jimenez, J., White-Cooper, H., Dawson, I., Nurse, P., Glover, D.M., 1992. *twine*, a cdc25 homolog that functions in the male and female germline of Drosophila. Cell 69, 977–988. http://dx.doi.org/10.1016/0092-8674(92)90616-K.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. 25, 3389–3402. http://dx.doi.org/10.1093/nar/25.17.3389.

Brook, M., Smith, J.W.S., Gray, N.K., 2009. The DAZL and PABP families: RNA-binding proteins with interrelated roles in translational control in oocytes. Reproduction 137, 595–617. http://dx.doi.org/10.1530/REP-08-0524.

Bergner, L.M., Hickman, F.E., Wood, K.H., Wakeman, C.M., Stone, H.H., Campbell, T.J., Lightcap, S.B., Favors, S.M., Aldridge, A.C., Hales, K.G., 2010. A novel predicted bromodomain-related protein affects coordination between meiosis and spermiogenesis in *Drosophila* and is required for male meiotic cytokinesis. DNA Cell Biol. 29, 487–498. http://dx.doi.org/10.1089/dna.2009.0989.

Castrillon, D.H., Gonczy, P., Alexander, S., Rawson, R., Eberhart, C.G., Viswanathan, S., Dinardo, S., Wasserman, S.A., 1993. Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: characterization of male-sterile mutants generated by single *P* element mutagenesis. Genetics 135, 489–505.

Cheng, M.H., Maines, J.Z., Wasserman, S.A., 1998. Biphasic subcellular localization of the DAZL-related protein Boule in *Drosophila* spermatogenesis. Dev. Biol. 204, 567–576. http://dx.doi.org/10.1006/dbio.1998.9098.

Courtot, C., Fankhauser, C., Simanis, V., Lehner, C.F., 1992. The *Drosophila* cdc25 homolog *twine* is required for meiosis. Development 116, 405–416.

Crozier, R.H., 1977. Evolutionary genetics of the Hymenoptera. Annu. Rev. Entomol. 22, 263–288. http://dx.doi.org/10.1146/annurev.en.22.010177.001403.

Cruz-Landim, C., Beig, D. Silva De, Moraes, R.L.M., 1980. The process of differentiation during spermatogenesis in bees (Hymenoptera, Apidae). Caryologia 33, 1–15. http://dx.doi.org/10.1080/00087114.1980.10796814.

Eberhart, C.G., Maines, J.Z., Wasserman, S.A., 1996. Meiotic cell cycle requirement for a fly homologue of human *Deleted in Azoospermia*. Nature 381, 783–785. http://dx.doi.org/10.1038/381783a0.

Eirin-Lopez, J.M., Ausio, J., 2011. Boule and the evolutionary origin of metazoan gametogenesis: a grandpa's tale. Int. J. Evol. Biol., Article ID: 972457, http://dx.doi.org/10.4061/2011/972457.

Fujiwara, Y., Akita, K., Okumura, W., Kodaka, T., Tomioka, K., Naito, T., 2004. Estimation of allele numbers at the sex-determining locus in a field population of the turnip sawfly (*Athalia rosae*). J. Hered. 95, 81–84. http://dx.doi.org/10.1093/jhered/esh009.

Hatakeyama, M., Nishimori, Y., Oishi, K., 2000. Participation of testicular spermatids in development upon intracytoplasmic injection into eggs of the sawfly, *Athalia rosae* (Insecta, Hymenoptera). J. Exp. Zool. 286, 181–192.

Hoage, T.R., Kessel, R.G., 1968. An electron microscope study of the process of defferentiation during spermatogenesis in the drone honey bee (*Apis mellifera* L.) with special reference to centriole replication and elimination. J. Ultrastruct. Res. 24, 6–32. http://dx.doi.org/10.1016/S0022-5320(68)80014-0.

Hogge, M.A.F., King, P.E., 1975. The ultrastructue of spermatogenesis in *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae). J. Submicrosc. Cytol. 7, 81–96.
Houston, D.W., King, M.L., 2000. A critical role for *Xdazl*, a germ plasm-localized RNA, in the differentiation of primordial germ cells in *Xenopus*. Development 127, 447–456.
Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755. http://dx.doi.org/10.1093/bioinformatics/17.8.754.
Joiner, M.L.A., Wu, C.F., 2004. Nervous system function for the testis RNA-binding protein Boule in *Drosophila*. J. Neurogenet. 18, 341–363. http://dx.doi.org/10.1080/01677060490477435.
Karashima, T., Sugimoto, A., Yamamoto, M., 2000. *Caenorhabditis elegans* homologue of the human azoospermia factor *DAZ* is required for oogenesis but not for spermatogenesis. Development 127, 1069–1079.
Karlsson-Rosenthal, C., Millar, J.B.A., 2006. Cdc25: mechanisms of checkpoint inhibition and recovery. Trends Cell Biol. 16, 285–292. http://dx.doi.org/10.1016/j.tcb.2006.04.002.
Kuales, G., De Mulder, K., Glashauser, J., Salvenmoser, W., Takashima, S., Hartenstein, V., Berezikov, E., Salzburger, W., Ladurner, P., 2011. *Boule*-like genes regulate male and female gametogenesis in the flatworm *Macrostomum lignano*. Dev. Biol. 357, 117–132. http://dx.doi.org/10.1016/j.ydbio.2011.06.030.
Lin, T., Viswanathan, Y., Wood, S., Wilson, C., Wolf, P.G., Fuller, M.T., 1996. Coordinate developmental control of the meiotic cell cycle and spermatid differentiation in *Drosophila* males. Development 122, 1331–1341.
Lin, Y., Page, D.C., 2005. Dazl deficeincy leads to embryonic arrest of germ cell development in XY C57BL/6 mice. Dev. Biol. 288, 309–316. http://dx.doi.org/10.1016/j.ydbio.2005.06.032.
Mable, B.K., Otto, S.P., 1998. The evolution of life cycles with haploid and diploid phases. Bioessays 20, 453–462.
Maegawa, S., Yamashita, M., Yasuda, K., Inoue, K., 2002. Zebrafish DAZ-like protein controls translation via the sequence 'GUUC'. Genes Cells 7, 971–984. http://dx.doi.org/10.1046/j.1365-2443.2002.00576.x.
Maegawa, S., Yasuda, K., Inoue, K., 1999. Maternal mRNA localization of zebrafish *DAZ*-like gene. Mech. Dev. 81, 223–226. http://dx.doi.org/10.1016/S0925-4773(98)00242-1.
Maines, J.Z., Wasserman, S.A., 1999. Post-transcriptional regulation of the meiotic Cdc25 protein Twine by the Dazl orthologue Boule. Nat. Cell Biol. 1, 171–174. http://dx.doi.org/10.1038/11091.
Mikhaylova, L.M., Boutanaev, A.M., Nurminsky, D.I., 2006. Transcriptional regulation by Modulo integrates meiosis and spermatid differentiation in male germ line. Proc. Natl. Acad. Sci. USA 103, 11975–11980. http://dx.doi.org/10.1073/pnas.0605087103.
Millar, J.B.A., Russell, P., 1992. The cdc25 M-phase inducer: an unconventional protein phosphatase. Cell 68, 407–410, http://dx.doi.org/10.1016/0092-8674(92)90177-E.
Naito, T., Suzuki, H., 1991. Sex determination in the sawfly, *Athalia rosae ruficornis* (Hymenoptera): occurrence of triploid males. J. Hered. 82, 101–104.
Nishiyama, T., Tachibana, K., Kishimoto, T., 2010. Cytostatic arrest: post-ovulation arrest until fertolization in metazoan oocytes. In: Verlhac, M.H., Villeneuve, A. (Eds.), Oogenesis. Wiley-Blackwell, West Sussex, UK, pp. 357–384.
Oishi, K., Sawa, M., Hatakeyama, M., Kageyama, Y., 1993. Genetics and biology of the sawfly, *Athalia rosae* (Hymenoptera). Genetica 88, 119–127. http://dx.doi.org/10.1007/BF02424468.
Pozarowski, P., Darzynkiewicz, Z., 2004. Analysis of cell cycle by flow cytometry. In Check Point Control and Cancer, vol. 2: activation and regulation protocols. In: Schonthal, A.H. (Ed.), Series Methods in Molecular Biology, vol. 281. Humana Press, Totowa, NJ, pp. 301–311.
Sawa, M., Fukunaga, A., Naito, T., Oishi, K., 1989. Studies on the sawfly, *Athalia rosae* (Insecta, Hymenoptera, Tenthredinidae). I. General biology. Zoo. Sci. 6, 541–547.
Sawa, M., Oishi, K., 1989. Studies on the sawfly, *Athalia rosae* (Insecta, Hymenoptera, Tenthredinidae). II. Experimental activation of mature unfertilized eggs. Zool. Sci. 6, 549–556.
Schurko, A.M., Logsdon, J.M., Eads, B.D., 2009. Meiosis genes in *Daphnia pulex* and the role of parthenogenesis in genome evolution. BMC Evol. Biol. 9, 78, http://dx.doi.org/10.1186/1471-2148-9-78.
Schurko, A.M., Mazur, D.J., Logsdon, J.M., 2010. Inventory and phylogenomic distribution of meiotic genes in *Nasonia vitripennis* and among diverse arthropods. Insect Mol. Biol. 19 (Suppl. S1), S165–S180. http://dx.doi.org/10.1111/j.1365-2583.2009.00948.x.
Shah, C., VanGompel, M.J.W., Naeem, V., Chen, Y., Lee, T., Angeloni, N., Wang, Y., Xu, E.Y., 2010. Widespread presence of human *BOULE* homologs among animals and conservation of their ancient reproductive function. Plos Genet. 6, e1001022. http://dx.doi.org/10.1371/journal.pgen.1001022.
Sharma, G.P., Gupta, B.L., Kumbkarni, C.G., 1961. Cytology of spermatogenesis in the honey-bee, *Apis indica* (F.). J. R. Microsc. Soc. 79, 337–351. http://dx.doi.org/10.1111/j.1365-2818.1961.tb05227.x.
Srinivasan, D.G., Fenton, B., Jaubert-Possamai, S., Jaouannet, M., 2010. Analysis of meiosis and cell cycle genes of the facultatively asexual pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae). Insect Mol. Biol. 19 (Suppl. S2), S229–S239. http://dx.doi.org/10.1111/j.1365-2583.2009.00960.x.
Sumitani, M., Yamamoto, D.S., Lee, J.M., Hatakeyama, M., 2005. Isolation of *white* gene orthologue of the sawfly, *Athalia rosae* (Hymenoptera) and its functional analysis using RNA interference. Insect Biochem. Mol. Biol. 35, 231–240. http://dx.doi.org/10.1016/j.ibmb.2004.12.006.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739. http://dx.doi.org/10.1093/molbev/msr121.
Tanabe, A.S., 2011. Kakusan4 and Aminosan: two programs for comparing nonpartitioned, proportional and separate models for combined molecular phylogenetic analyses of multilocus sequence data. Mol. Ecol. Resour. 11, 914–921. http://dx.doi.org/10.1111/j.1755-0998.2011.03021.x.
The Honeybee Genome Sequencing Consortium, 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443, 931–949. http://dx.doi.org/10.1038/nature05260.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680. http://dx.doi.org/10.1093/nar/22.22.4673.
VanGompel, M.J.W., Xu, E.Y., 2010. A novel requirement in mammalian spermatid differentiation for the DAZ-family protein Boule. Hum. Mol. Genet. 19, 2360––2369. http://dx.doi.org/10.1093/hmg/ddq109.
VanGompel, M.J.W., Xu, E.Y., 2011. The roles of the *DAZ* family in spermatogenesis: more than just translation? Spermatogenesis 1, 36–46. http://dx.doi.org/10.4161/spmg.1.1.14659.
Werren, J.H., Richards, S., Desjardins, C.A., Niehuis, O., Gadau, J., Colbourne, J.K., Group, T.N.G.W., 2010. Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. Science 327, 343–348. http://dx.doi.org/10.1126/science.1178028.
White-Cooper, H., 2010. Molecular mechanisms of gene regulation during *Drosophila* spermatogenesis. Reproduction 139, 11–21. http://dx.doi.org/10.1530/REP-09-0083.
White-Cooper, H., Alphey, L., Glover, D.M., 1993. The *cdc25* homologue *twine* is required for only some aspects of the entry into meiosis in *Drosophila*. J. Cell Sci. 106, 1035–1044.
Xu, E.Y., Moore, F.L., Reijo Pera, R.A., 2001. A gene family required for human germ cell development evolved from an ancient meiotic gene conserved in metazoans. Proc. Nat. Acad. Sci. USA 98, 7414–7419. http://dx.doi.org/10.1073/pnas.131090498.
Yamamoto, D.S., Tachibana, K., Sumitani, M., Lee, J.M., Hatakeyama, M., 2008. Involvement of Mos–MEK–MAPK pathway in cytostatic factor (CSF) arrest in eggs of the parthenogenetic insect, *Athalia rosae*. Mech. Dev. 125, 996–1008. http://dx.doi.org/10.1016/j.mod.2008.08.004.
Yen, P.H., 2004. Putative biological functions of the DAZ family. Int. J. Androl. 27, 125–129. http://dx.doi.org/10.1111/j.1365-2605.2004.00469.x.
Yoshiyama, N., Tojo, K., Hatakeyama, M., 2013. A survey of the effectiveness of non-cell autonomous RNAi throughout development in the sawfly, *Athalia rosae* (Hymenoptera). J. Insect Physiol. 59, 400–407. http://dx.doi.org/10.1016/j.jinsphys.2013.01.009.